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Dying two deaths — programmed cell death regulation in development and disease

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Programmed cell death (PCD) is a fundamental cellular process that has adopted a plethora of vital functions in multicellular organisms. In plants, PCD processes are elicited as an inherent part of regular development in specific cell types or tissues, but can also be triggered by biotic and abiotic stresses. Although over the last years we have seen progress in our understanding of the molecular regulation of different plant PCD processes, it is still unclear whether a common core machinery exists that controls cell death in development and disease. In this review, we discuss recent advances in the field, comparing some aspects of the molecular regulation controlling developmental and pathogen-triggered PCD in plants.

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Introduction

There is no life without death — in modern biology, this ancient axiom has proven to be of remarkable significance. In individual organisms, genetically encoded programs of ageing and death control the turnover of generations, which is the driver of adaptive evolution. Likewise, the genetically programmed death of cells (PCD) in multicellular organisms has acquired a multitude of crucial roles in development, homeostasis and immunity [1,2].

In plants, various forms of PCD have been described as an inherent part of development, as well as a response to biotic and abiotic stresses. Developmentally controlled PCD (dPCD) occurs during vegetative and reproductive development, often as the final differentiation step of specific cell types; it ends the vital function of senescing or no longer required cells, or creates tissues composed of modified cell corpses that take over structural or storage functions [3]. On the other hand, pathogen-triggered PCD (pPCD) can be elicited in the host plant by invading agents. However, depending on the type of plant–pathogen interaction, pPCD will benefit either the plant or the pathogen [4]. Invasion of biotrophic or hemibiotrophic pathogens — those that feed exclusively or at early stages of their life cycle on live plant tissue — can be thwarted by pathogen detection, triggering hypersensitive response (HR) cell death at the site of attempted attack. In contrast, necrotrophic pathogens, which feed on dead plant tissue, have often developed strategies to silently invade the host plant and hijack its HR machinery, triggering unrestrained PCD at the site of infection and beyond.

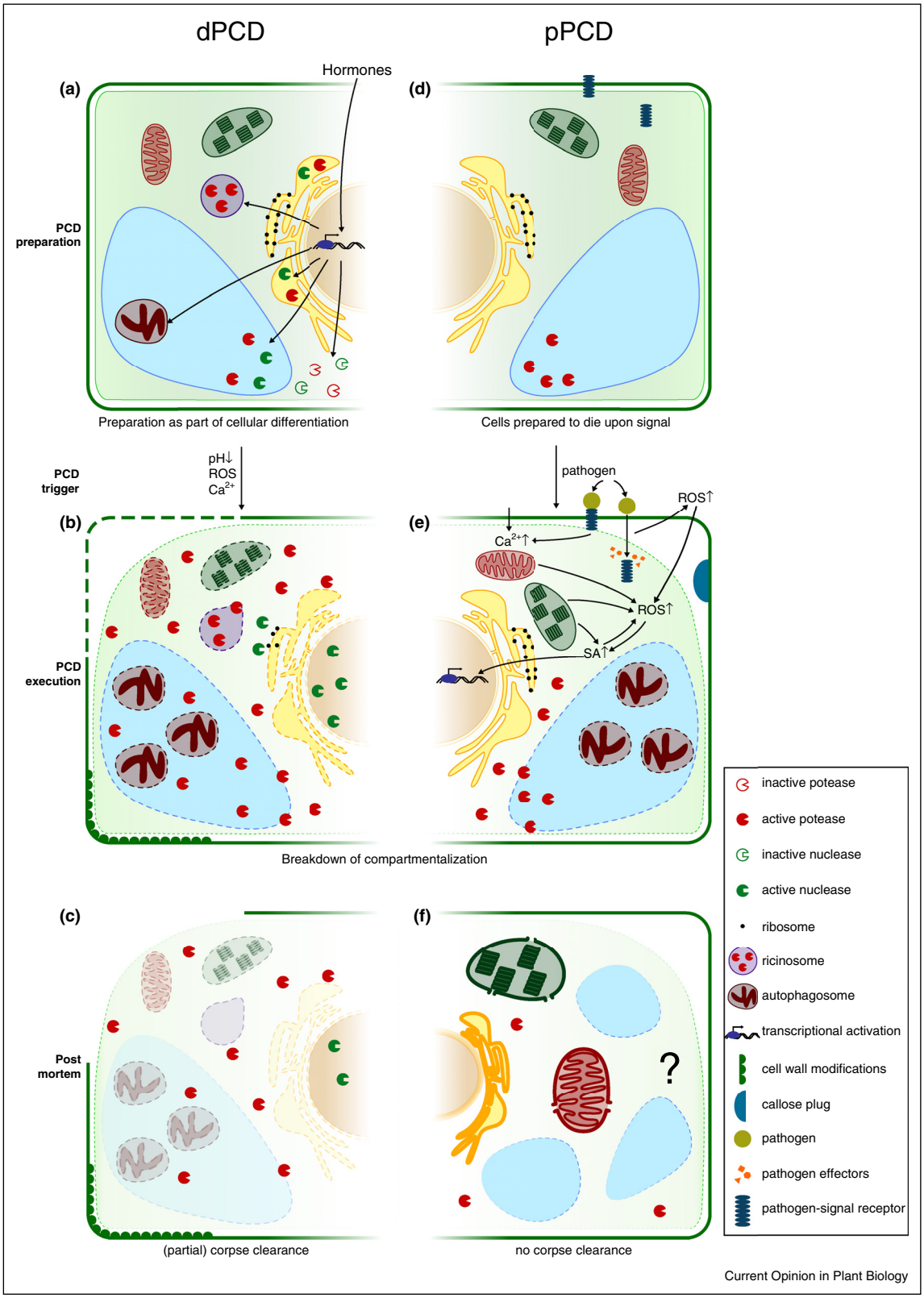
Morphologically, dPCD is associated with a vacuolar type of cell death, while pPCD shows features of both necrosis and vacuolar PCD [5]. However, the molecular regulation of PCD initiation and execution in development and disease remains largely unresolved. Especially the intriguing question of whether dPCD and pPCD are controlled by a common core machinery or by fundamentally different pathways is a matter of debate. In this review, we will highlight the recent advances in dPCD and pPCD research, focusing on comparing the molecular regulation of these different PCD types in plants.

The molecular regulation of dPCD

Hormonal signaling during dPCD

Different hormonal pathways are interconnected to fine-tune dPCD processes (Figure 1a). For instance jasmonic acid, ethylene, auxin and strigolactones have been implicated in dPCD signaling, although exact networks are often still unknown [6–8]. Among them, ethylene is the best-characterized dPCD hormone. In the lace plant (*Aponogetum madagascariensis*), increased ethylene levels, and decreased expression of repressive AmERS1 ethylene receptors is associated with PCD in specific leaf regions to create perforations [9]. After fertilization in Arabidopsis (*Arabidopsis thaliana*), ethylene signaling contributes to the elimination of the persistent synergid via cell fusion and nuclear degradation, terminating pollen tube attraction [10•,11]. In xylogenic cell cultures of

Figure 1



Chronological overview of the different molecular steps during dPCD and pPCD. (a) to (c) show dPCD events. **(a)** dPCD preparation as a part of cellular differentiation is initiated by hormonal signaling. This leads to transcriptional activation of dPCD genes, like proteases and nucleases,

Zinnia elegans, chemical inhibition of ethylene signaling delays xylem differentiation, but also directly blocks PCD [12]. This finding indicates that hormones can control both upstream differentiation events as well as downstream dPCD execution.

Transcriptional preparation of dPCD

Plant hormones control many cellular processes via transcriptional regulation [13], including differentiation and dPCD (Figure 1a), although the connection between hormones and transcription factors (TFs) is often still missing. PCD as final differentiation step of certain cell types has to be tightly coordinated with earlier differentiation steps, as precocious or delayed PCD can severely interfere with cellular functions (see [3] for a recent review). NAC (NAM, ATAF and CUC) TFs are one of the most-studied TF families in this context. ORESARA1 (ANAC092) is a master regulator of leaf senescence downstream of ethylene, and upstream of genes that induce senescence and PCD, including BIFUNCTIONAL NUCLEASE 1 (BFN1) and other NAC TFs [14,15•]. Similarly, SOMBRERO (SMB/ANAC033) controls dPCD as a final step of lateral root cap (LRC) differentiation in Arabidopsis [16••]. In the *smb* mutant, LRC cells die in an aberrant, non-prepared fashion, and cell corpses remain non-degraded on the root surface. During xylem differentiation, VASCULAR-RELATED NAC DOMAIN 7 (ANAC030) is part of a complex transcriptional network that induces expression of downstream TFs and putative PCD executers [17].

Other TF families have also been implicated in dPCD control. In the receptive synergid of Arabidopsis, the two reproductive meristem TFs VERDANDI and VALKYRIE are directly activated by the MADS-box TF complex SEEDSTICK-SEPALLATA 3 to regulate synergid degeneration [18], a prerequisite for successful fertilization. After fertilization, the endosperm-expressed MADS-box TF AGAMOUS-LIKE 62 triggers PCD in the adjacent nucellus via an unknown signal that activates the PCD-promoting MADS-box TFs TRANSPARENT TESTA 16 and GORDITA [19•]. During mid-seed development, endosperm degeneration is initiated by a heterodimer of two endosperm-expressed bHLH TFs, ZHOUPI (ZOU) and INDUCER OF CBP EXPRESSION 1 [20]. In the *zou* mutant, embryo growth is hampered by a persistent rigid endosperm, associated with reduced expression of cell wall modifying enzymes, indicating that cell wall degradation might be a mechanical prerequisite for endosperm PCD [21].

Triggers of dPCD

The gradual buildup of dPCD competence in the course of cellular differentiation stands in contrast to the rapidly triggered execution of cell death. Several cellular signals, including calcium fluxes, accumulation of reactive oxygen species (ROS), and cytoplasmic acidification have been implicated in PCD triggering [22] (Figure 1b).

Calcium signaling is involved in many cellular processes [23], including PCD. During the self-incompatibility (SI) response in poppy (*Papaver rhoeas*), calcium influx triggers a signaling cascade that induces rapid PCD of the incompatible pollen tubes [24•]. In Arabidopsis ovules, fertilization requires coordinated disintegration of the pollen tube and the synergid cell. A calcium dialogue in both cells has been observed, and aberrant calcium signatures in the synergid obstruct pollen tube burst and synergid PCD [25••,26••].

ROS have been suggested to play a role in stress responses as well as dPCD. High levels of ROS can directly kill a cell by causing membrane leakage [27], whereas lower levels of ROS can have diverse signaling functions [22]. In the rice *dtc1* mutant, tapetum PCD is delayed due to a failure of ROS accumulation [28•]. Altering ROS production via manipulation of RESPIRATORY BURST OXIDASE HOMOLOG E disturbs timing of tapetal PCD in Arabidopsis [29]. In the poppy SI response, ROS accumulate in the pollen tube [30], possibly to control pollen tube burst by cell wall remodeling, and prior to sperm delivery in Arabidopsis, ROS induce pro-PCD protease activity [31].

Finally, cytoplasmic acidification has been implicated in dPCD processes. The SI response in poppy causes a dramatic pH drop that is necessary and sufficient to activate several proteases, and to induce PCD [24•]. Also during LRC PCD in Arabidopsis, acidification of the cytoplasm was observed prior to cell death, and manipulation of intracellular pH affected cell death rates [16••].

dPCD execution and corpse clearance

Upon triggering signals, PCD execution and *post mortem* corpse clearance are initiated (Figure 1c). A multitude of lytic enzymes is activated or released from safe storage

(Figure 1 Legend Continued) which are sequestered or kept inactive. Only upon a cell death trigger, like calcium, ROS or pH drop, PCD execution is initiated. **(b)** During dPCD execution, lytic enzymes are activated or released from safe storage and degrade the various cellular compartments, and in the xylem, cell walls are fortified. Upregulation of autophagy can occur. **(c)** At the end of dPCD, the cell corpse is completely degraded, or only the fortified cell wall remains. (d) to (f) show pPCD events. **(d)** pPCD is only triggered upon pathogen attack, mediated by receptors present on the membrane or in the cytoplasm of all cells of a plant. **(e)** When a pathogen invades a plant cell, the activated receptor increases calcium and ROS levels in the cell, leading to the production of salicylic acid (SA). SA, in turn, induces transcription of pPCD related genes, and amplifies the ROS burst in a positive feedback loop, creating a toxic environment. **(f)** The exact mechanisms of cellular degradation during pPCD are still largely unknown, but complete cell corpse clearance is absent. The cells undergo vacuolization and the organelles swell and burst.

compartments to degrade cellular components [22]. Dying Arabidopsis LRC cells for instance are completely degraded via a cell-autonomous program controlled by SMB [16••]. In xylem cells, however, only the protoplast is degraded, while a fortified cell wall remains, fulfilling essential *post mortem* tasks in water transport and wood formation [32].

During corpse clearance, nucleic acid species are degraded. Although nuclear degradation is frequently reported [28•,29,33•,34•] only few molecular players have been identified. In the LRC of Arabidopsis, BFN1 is responsible for DNA degradation, because the *bfn1* mutant exhibits non-degraded nuclear remnants at the root surface. To allow a safe BFN1 production in living cells, this protein is only released from the endoplasmic reticulum (ER) upon PCD initiation [16••].

Besides nucleases, proteases are also involved in PCD execution and corpse clearance [22]. In tomato endosperm and the Arabidopsis root cap, cysteine proteases are stored in ER-derived compartments [35,36], while in the Arabidopsis tapetum, they are transported to the vacuole [33•]. For several proteases, caspase-like activities were found, for instance vacuolar processing enzymes (VPEs) or certain subunits of the proteasome [37] (for a recent overview of caspase-like activities in dPCD, see [22]). Despite the detection of caspase-like activities, their precise functions remain largely mysterious. On the other hand, the distantly caspase-related metacaspases (MCs) do not possess a caspase-like activity, and some of them have been implicated in dPCD. For instance, MC9 in Arabidopsis has been implicated in corpse clearance during xylem PCD [38]. Interestingly, independent findings suggest a connection between MCs and autophagy. MC9 in the tracheary elements (TEs) might have an additional *pre mortem* function in reducing autophagy levels to protect the surrounding cells [39]. Contrarily, in the spruce suspensor, mcII-Pa promotes autophagy, which is necessary for a controlled PCD execution and prevents the switch to a necrotic form of cell death [40].

The molecular regulation of pPCD

Hormonal signaling during pPCD

Plant hormones are crucial for plant immune responses, controlling complex and pathosystem-specific networks determining the outcome of a particular plant–pathogen interaction. Among them, SA is the only phytohormone strictly required for the establishment of pPCD. SA promotes pPCD leading to immunity against biotrophs and susceptibility towards necrotrophs [41,42]. Tightly regulated positive feedback loops between SA and ROS are essential to ensure rapid amplification of defense responses [43] (Figure 1e).

Considering the importance of SA signaling, it is not surprising that biotrophic/hemibiotrophic pathogens have evolved strategies to subvert the SA signaling pathway as a virulence strategy. Some pathogens deliver effector proteins that directly interfere with cellular SA biosynthesis or signaling [4]. Alternatively, some pathogens suppress SA-mediated defenses by producing phytotoxins that tamper with the crosstalk between SA and other hormones involved in immunity. This is the case for coronatine from *Pseudomonas syringae*, which mimics the SA antagonist jasmonic acid [44•,45,46]. Another example is PSE1 from *Phytophthora parasitica*, a toxin that promotes auxin accumulation at infection sites, resulting in inhibition of SA-mediated cell death and increased pathogen growth [47].

Triggers of pPCD

Cytoplasmic immune receptor-mediated recognition at the site of attack has been considered as the main pPCD trigger during plant-biotrophic/hemibiotrophic pathogen interactions [48] (Figure 1d). In fact, pPCD phenotypes can be triggered by autoactivation of many different cytoplasmic immune receptor proteins and can be suppressed by removal of SA or inhibition of SA signaling pathways [49,50]. Membrane-associated immune receptor-like kinases (RLKs) can also regulate cell death. This is the case of BIR1, a suppressor of plant defense whose inactivation triggers pPCD mediated by association of two additional immune RLKs: SOBIR and BAK1 [51••]. In fact, the importance of the apoplast in pPCD has just started to emerge, as is the source of many potential pPCD triggers like RLK ligands, ROS, nitric oxide (NO) and proteases.

It is well established that pathogen perception triggers calcium influxes, as well as accumulation of SA, ROS and NO. SA signaling is preceded by oxidative bursts originating in different cellular compartments, but ROS acts also downstream of SA [52]. This positive SA–ROS feedback loop can be considered as a pPCD trigger, although the molecular details of this activation remain to be elucidated (Figure 1e).

The pPCD machinery has been conveniently hijacked by plant necrotrophic pathogens, some of which are able to secrete pPCD triggering toxins. A good example is the fungus *Cochliobolus victoriae*, which secretes victorin into host cells. This results in the activation of the cytoplasmic immune receptor LOV1, which causes pPCD and susceptibility to *C. victoriae* [53]. Another toxin with PCD-triggering activity is oxalic acid from the necrotrophic fungus *Sclerotinia sclerotiorum*. Oxalic acid deficiency renders *S. sclerotiorum* non-pathogenic, inducing autophagy-mediated cell death and various defense responses in the host [54,55].

Regulation, execution and confinement of pPCD

Transcriptional regulation during dPCD and pPCD are markedly different. A transcriptomic meta-analysis

revealed several clusters of genes providing unique transcriptional signatures for different plant PCD types. However, in the case of pPCD, the cluster identified includes a set of genes most of which are involved in defense, rather than specifically in pPCD [34•]. Nevertheless, TFs play essential roles in the establishment of immune responses in plants [56]. The best understood TF promoting pPCD and defense responses is undoubtedly Arabidopsis MYB30. MYB30 is involved in the SA amplification loop that controls pPCD. It also regulates the biosynthesis of very long chain fatty acids, precursors of lipid derivatives with roles in cell death signaling and basal defense [57].

Calcium has been proposed as a master regulator that contributes to triggering pPCD and ensures its timely and controlled execution [58]. Blocking calcium transport by $LaCl_3$ or ruthenium red inhibits pPCD [59]. The calcium-dependent protein kinases CPK1 and *2* have been shown to specifically regulate the onset of pPCD together with CPK5 and 6, which phosphorylate and activate various WRKY TFs [59]. Calcium also acts as a negative regulator of SA signaling presumably to shut down defenses when they are no longer needed [60]. In addition, a calcium-binding protein and a calcium-regulated ATPase have been identified as part of the meta-transcriptomic pPCD cluster [34•].

Autophagy can act as a positive or negative regulator of pPCD depending on the pathosystem [55,61••,62]. The Arabidopsis metacaspase AtMC1 acts synergistically with autophagy to promote pPCD [63••]. Similarly, retromer-mediated vacuolar trafficking has been shown to be required for defense and pPCD [64•]. Wheat metacaspase 4 (*TaMCA4*) overexpression enhances pPCD caused by effector-mediated recognition of the hemibiotrophic fungus *Puccinia striiformis* and contributes to disease resistance, whereas its silencing causes the opposite effect [65]. Several additional regulators have recently emerged as key for a proper establishment of pPCD. VPEs, phytaspase and saspase have been shown to be the most important sources of caspase-like activities involved in pPCD [66], although their individual contribution may vary depending on the specific pathosystem.

Equally important as positive regulation for pPCD establishment are negative regulators to confine the damage to the cells destined to die. Autophagy has been shown to prevent runaway pPCD [67]. AtMC1-mediated pPCD is negatively regulated by AtMC2 and AtLSD1 [68]. AtLSD1 function is partly mediated by its SA-dependent interaction with catalases, which have been proposed to prevent runaway cell death by modulating ROS accumulation [69]. Unfortunately, most studies carried out to date lack the spatio-temporal dimension of the interaction. It has been long assumed that positive regulators act at the HR site and negative regulators in the surrounding areas, but the molecular evidence for this premise is mostly lacking and the functional zonation of pPCD remains to be clarified.

Conclusions

Among the various types of plant PCD, several distinct forms of dPCD and pPCD have been studied over the last years. Despite recent progress in identifying PCD regulators and in understanding their molecular mode of action, it remains hard to fathom whether dPCD and pPCD share canonical, evolutionary conserved core PCD regulators, or whether similarities are merely mechanistic parallels that have been independently adopted to fulfill analogous roles in the different contexts.

Undoubtedly, there are numerous similarities that can be observed in dPCD and pPCD. ROS and calcium have been implicated in signaling events leading to cell death in both contexts. Metacaspases have been assigned different roles in dPCD and pPCD, from upstream regulation to downstream *post mortem* cell clearance. Other proteases, for instance the VPEs with caspase-like activity, are involved in dPCD and pPCD processes as well [37]. Likewise, modulation of autophagy has been functionally implicated in both forms of PCD; as an effector of pPCD and as a corpse clearance mechanism during dPCD [70].

There is also common evidence of transcriptional regulation, though within different contexts. In many dPCD forms, cells need to gradually acquire a competence to execute cell death upon specific developmental signals. In contrast, cells always need to be ready to initiate immune responses upon pathogen attack independent of their cellular identity (Figure 1a,d). In order to be of selective advantage, transcriptional responses have to be rapid and direct to counteract pathogen attack, with death being sometimes unavoidable, but beneficial for the whole organism, as it has been conserved through evolution.

In a way, forms of pPCD can be regarded as a facultative outcome of signaling processes between different cells that come into contact (host and pathogen), and are in that way similar to some forms of dPCD that involve signaling between different cell types. For instance, poppy pollen dies only when contacting stigmatic papilla cells that express the cognate ('self') S-determinant [30]. Similarly, pollen and synergid cells only die in a controlled way after establishing an elaborate calcium dialogue [25••,26••]. Possibly these facultative non-cell autonomous forms of dPCD are more closely related to forms of pPCD than autonomous forms of differentiation-induced dPCD. Interestingly, the RLK FERONIA promotes both pollen tube reception as well as susceptibility to powdery mildew infection [71], corroborating the existence of molecular links between developmentally controlled and pathogen-related forms of PCD. More such regulators with dual roles in dPCD and pPCD may be expected to see the light in the near future of PCD research.

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